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14. ABSTRACT
The tumor suppressor BRCA1 has been implicated in numerous cellular processes, including cell cycle checkpoint control, DNA repair, and mitotic spindle assembly. In vivo, BRCA1 primarily exists in association with BARD1, and the BRCA1/BARD1 heterodimer is thought to mediate the tumor suppression activity of BRCA1. It has been shown that the phosphorylation state of the BARD1 polypeptide is cell- cycle regulated and that BARD1 is hyperphosphorylated in mitosis at seven distinct residues. To study the function of mitotic BARD1 phosphorylation, an siRNA-mediated approach was employed to knockdown endogenous BARD1 expression. In this manner, I evaluated the role of BARD1 in clonogenic survival following genotoxic stress, DNA damage-induced cell cycle checkpoints, mitotic spindle assembly, and homology-directed repair (HDR). Knockdown of BARD1 resulted in a dramatic decrease in survival in response to IR, mitomycin C (MMC) and camptothecin (CPT). Rescue with an siRNA-resistant wild-type BARD1 construct resulted in a substantial increase in cell survival; however, reconstitution with siRNA-constructs bearing point mutations at all seven sites produced a decrease in survival in response to MMC and CPT, suggesting an important role for BARD1 mitotic phosphorylations in response to certain forms of damage. Knockdown of BARD1 resulted in substantial defects in both the IR-induced G2 and transient G2/M checkpoints, indicating a role for BARD1 in these processes. Rescue with phosphomutant forms of BARD1 bearing siRNA –resistance did not appear to result in defect in the G2 accumulation checkpoint, suggesting that mitotic phosphorylations do not function in this role. While BARD1 does not appear to function in the mitotic exit checkpoint or spindle assembly checkpoint, it does have a role in proper mitotic spindle assembly. Reconstitution experiments are currently underway to determine the role of mitotic phosphorylations in the process of mitotic spindle assembly. To examine the role of BARD1 phosphorylations in HDR, a Bard1-null mammary carcinoma cell line was reconstituted with wild-type BARD1 and both phosphomutant and phosphomimicking mutant forms of the protein. Experimental data shows that wild-type, phosphomutant, and phosphomimicking forms of BARD1 rescue the HDR defect in Bard1-nulls cells comparably, suggesting that mitotic phosphorylations do not play a role in this repair process.

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INTRODUCTION

The objective of my research is to study the role of BRCA1 in breast cancer by determining how BARD1 phosphorylation affects the checkpoint and DNA repair functions of the BRCA1/BARD1 heterodimer [1].

In my original application, I proposed to examine the role of BARD1 phosphorylation in the checkpoint functions of BRCA1 by generating and characterizing isogenic subclones of HCT116 cells that express different knock-in alleles of BARD1. Subsequently, however, I also tested the feasibility of an alternative approach based on siRNA-mediated depletion of endogenous BARD1 coupled to transient reconstitution with exogenous BARD1. This approach has several advantages over the original knock-in strategy. First, since it involves transient transfection of a cell population, this approach is not susceptible to artifacts that arise due to clonal variation. Second, unlike the knock-in strategy, which is restricted to certain pseudo-diploid cell lines such as HCT116, this approach can be applied to a broad range of cell types. Third, this approach is more facile since it does not require the laborious process of generating stable knock-in subclones by targeted gene recombination.

The siRNA-mediated approach was optimized and employed to determine the role of BARD1 itself and BARD1 phosphorylation in the known BRCA1-dependent cell cycle checkpoints, including the IR-induced transient G_2/M checkpoint, IR-induced G_2 accumulation checkpoint, IR-induced mitotic exit checkpoint and the spindle assembly checkpoint. Cell sensitivity to DNA damaging agents, including IR, mitomycin C (MMC), and camptothecin (CPT), was analyzed by the siRNA-mediated system as well. Lastly, since BRCA1 was recently reported to have an essential role in mitotic spindle assembly [2], the role of BARD1 and mitotic BARD1 phosphorylation this process in was also characterized by siRNA knockdown.

To evaluate the role of BARD1 phosphorylation in homology-directed repair (HDR) of double-strand DNA breaks (DSB), Bard1-null mouse mammary tumor cells bearing an HDR reporter construct (DR-GFP) were transfected with expression vectors encoding either wildtype or mutant forms of human BARD1 [3]. Briefly, this reporter contains two distinct nonfunctional copies of the GFP gene: one copy (*Sce*GFP) is disrupted by the recognition site for the rare-cutting endonuclease I-*Sce*I, while the other copy (*i*GFP) encodes only an internal region of GFP. However, a functional GFP gene can be regenerated when a DSB break triggered by I-*Sce*I cleavage of SceGFP is repaired by HDR utilizing *i*GFP as a template, and such events can be quantified by flow cytometry. Using this assay, we previously showed that transfection of these Bard1-null cells with an expression vector encoding human BARD1 induces an approximately 5-fold increase in HDR function [3]. This approach allowed me to determine if BARD1 phosphorylation mutations impair BARD1 function in HDR.

BODY

The checkpoint functions of BRCA1

To implement the siRNA-mediated approach to examine checkpoint function, I first designed two distinct siRNAs (siRNAs A and B) that can greatly reduce endogenous BARD1 expression (>90%) in a variety of cell lines (Figure 1). Second, by site-directed mutagenesis I introduced non-coding mutations into our BARD1 mammalian expression plasmids that render the resultant mRNAs resistant to knockdown by either siRNA. With these reagents, we should be able to test whether BARD1 phosphorylation is required for specific checkpoint functions of BRCA1. For example, a BRCA1-dependent function, such as the IR-induced G_2 accumulation checkpoint, should be ablated by siRNA-mediated BARD1 knockdown, either as a direct consequence of BARD1 inactivation or as an indirect consequence of BRCA1 instability in the absence of BARD1. In either case, transfection of the siRNA-treated cells with a siRNA-resistant vector encoding wildtype BARD1 should rescue the checkpoint. If, however, a specific BARD1 phosphorylation site (for example, S251) is required for the G_2 accumulation checkpoint, then transfection with a siRNA-resistant vector encoding S251A-mutant BARD1 should restore the expression levels of BRCA1 but not rescue checkpoint activity. Thus, by reconstituting siRNA-treated cells with siRNA-resistant expression vectors encoding the full panel of wildtype and phosphorylation site mutant BARD1 polypeptides, we should be able to identify the precise requirements for BARD1 phosphorylation in checkpoint function. A similar strategy was used successfully by Yu *et al.* to demonstrate a requirement for BACH1 phosphorylation in the same IR-induced G_2 accumulation checkpoint [4]. Moreover, this strategy was used not only to study the G_2 accumulation checkpoint (Task 1), but also a variety of other checkpoints including the IR-induced transient G_2/M, the IR-induced mitotic exit, and the spindle assembly checkpoint (Task 2).

<u>The IR-induced G_2 accumulation checkpoint</u>: In evaluating the effect of BARD1 knockdown on the G_2 accumulation checkpoint in 293 cells, siRNA-mediated knockdown of BRCA1 was included as a positive control, since BRCA1 is known to be required for this checkpoint [4]. Approximately 48 hrs and 72 hrs post-second transfection, one set of cells was irradiated with 10 Gy, while a second set was mock treated. After three hours at 37 ^{o}C, both treated and mock-treated cells were incubated for 15 hours with nocodazole (1 ug/mL) to arrest cells in prometaphase of mitosis. The cells were then fixed with 70% ethanol and placed at -20^{o}C overnight. The mitotic population of each culture was then measured by flow cytometric analysis after staining with propidium iodide and the mitotic marker, phospho-histone H3. As expected, knockdown of BRCA1 caused a defect in activation of the G_2 accumulation checkpoint. Significantly, BARD1 knockdown also induced a checkpoint defect, as illustrated by an ~5-10 fold

increase in the percentage of BARD1-depleted cells that entered mitosis following IR treatment relative to control cells (Figure 2).

To confirm that the observed checkpoint defect is due to BARD1 knockdown, and not due to non-specific off-target effects of the siRNAs, we introduced silent mutations into the siRNA-specific targeting regions of a BARD1 mammalian expression vector to render its mRNA product resistant to either the BARD1-specific siRNA A or B. Two mutations, especially if placed together near the middle of the siRNA sequence, are generally sufficient to ablate siRNA-mediated knockdown, although more mutations can only help [5]. In our design of siRNA-resistant BARD1 expression vectors, we were able to introduce 3 or 4 tandem nucleotide changes that disrupted siRNA complementarity but did not alter the coding potential of the vector. Of note, the BARD1 polypeptides encoded by these vectors contain an N-terminal tag of three tandem FLAG epitopes that allows the endogenous and exogenous (i.e., vector-encoded) forms of BARD1 to be distinguished in rescue experiments. To ascertain whether the G_2 accumulation checkpoint of the BARD1 siRNA-treated cells is due to BARD1 depletion, 293 cells that had been BARD1-depleted by two successive siRNA transfections (with siRNAs A or B) were transiently co-transfected with the appropriate siRNA-resistant BARD1 expression vector. Western blot analysis with a FLAG-specific antibody confirmed successful expression of exogenous BARD1 in siRNA-treated cells (Figure 3). Significantly, these cells displayed an approximately 5-fold decrease in the percentage of mitotic cells after IR exposure, indicating that reconstitution of BARD1-depleted cells with siRNA-resistant wildtype BARD1 provides a rescue of the G_2 accumulation checkpoint (Figure 4). Notably, reconstitution of the BARD1-depleted cells with siRNA-resistant BARD1 polypeptides bearing specific mitotic phosphorylation mutations resulted in an intact G_2 accumulation checkpoint, suggesting that mitotic phosphorylation of BARD1 is not required for this checkpoint (Figure 4).

The IR-induced transient G_2/M checkpoint: In previous studies, siRNA-mediated depletion in Hela cells has been used successfully to implicate the CtIP and BRCA1 proteins in the transient G_2/M cell cycle checkpoint [6]. The IR-induced transient G_2/M checkpoint is distinct from the G_2 accumulation checkpoint in that it occurs shortly after IR-damage (1-2 hrs) and it is a dose-independent checkpoint [7]. In our studies, two rounds of siRNA transfections performed approximately 24 hrs apart led to efficient knockdown of BARD1 protein expression (Figure 5). One set of cells was irradiated with 5 Gy, while a second set was mock treated. After one hour at 37 ^{o}C, the cells were fixed with 70 % ethanol and placed at -20 ^{o}C overnight. The mitotic population of each sample was then measured by flow cytometric analysis after staining with propidium iodide and the mitotic marker, phospho-histone H3. As expected, knockdown of CtIP caused a defect in activation of the transient G_2/M checkpoint [6] (Figure 6). Significantly, BARD1 knockdown also induced a checkpoint defect, as illustrated by ~3-5 fold increase in the percentage of cells that entered mitosis following IR treatment relative to control cells (Figure 6). However, attempts to rescue the

checkpoint by transient co-transfection with of siRNA-resistant cDNA were not effective in this cell line. A lentiviral approach was then taken to produce stable BARD1 siRNA-resistant cell lines under Blasticidin selection. While drug-resistant positive clones were successfully identified via western blotting techniques, the expression of exogenous Flag-tagged BARD1 polypeptides was extremely short-lived and thus, suitable stably-transformed clones could not be established for rescue assays. Thus, I am currently testing an alternative approach involving transient infection of siRNA-resistant lentiviruses encoding BARD1 siRNAs A and B (Figure 7) (Invitrogen; protocol as described by Yu et al 2003 [4]). Two lentiviral infections followed by two siRNA transfections will be carried out in He la cells prior to assessing the transient G_2/M checkpoint. Once rescue of the IR-induced transient G_2/M checkpoint is established with transient lentiviral infections, we will test viruses bearing phospho-mutant forms of BARD1 to determine the role of BARD1 mitotic phosphorylation in this checkpoint.

Spindle Assembly Checkpoint and Mitotic Exit Checkpoint: With these systems for depletion and reconstitution of BARD1 expression in place, we evaluated the role of BARD1 phosphorylation in additional checkpoints that are dependent on BRCA1: the spindle assembly checkpoint and the IR-induced mitotic exit checkpoint. It has been shown that a single unattached kinteochore is sufficient to activate the spindle checkpoint [8]. Treatment of cells with drugs such as paclitaxel and nocodazole activates the spindle assembly checkpoint in a BRCA1-dependent manner [9] causing cells to arrest in prometaphase of the cell cycle. Cells will not proceed to anaphase until all chromosomes are attached with their kinetochores to the microtubules of the spindle in the presence of an intact spindle assembly. To test the role of BARD1 in the spindle assembly checkpoint, 293 cells were depleted of BARD1 by two rounds of siRNA treatment (Figure 8). Limited data about the role of BRCA1 in the spindle checkpoint exists; nonetheless, since BRCA1 knockdown has been shown to result in a modest spindle assembly checkpoint defect in human cells [9], BRCA1 siRNA was utilized as a positive control. Next, cells were either mock treated or treated with 100 ng/mL nocodazole before harvesting at 12, 24, and 36 hr time points [10]. The mitotic population of each culture was then measured by flow cytometric analysis after staining with propidium iodide and the mitotic marker, phospho-histone H3. Our results indicate that, as expected, mock-treated cells show between ~2-4% of total population in mitosis (Figure 9). Cells treated with nocodazole, regardless of the siRNA treatment (control, BARD1 or BRCA1 siRNA), show comparable levels of cells in mitosis at all time points, with the effects of nocodazole treatment wearing off by 36 hrs with a concomitant reduction in mitotic levels (Figure 9). An analogous experiment was performed in cells utilizing paclitaxel, an anti-microtubule agent that also induces mitotic arrest. Untreated cells once again resulted in low mitotic levels (~2-4%); cells treated with paclitaxel, independent of the siRNA utilized, resulted in an intact spindle checkpoint with a comparable, high percentage of cells in mitosis at all time points, with the effects of the drug wearing off at 24 hours (Figure 10). Comparable experiments were carried out in Hela cells as well, resulting in

similar data of an intact spindle checkpoint despite efficient knockdown of BARD1 (data not shown). Therefore, based our data we can conclude that BARD1 is not required for a functional spindle checkpoint, in the presence of either paclitaxel or nocodazole drug treatments.

It was reported that BRCA1 may have a possible role in the IR-induced mitotic exit checkpoint [11]. To determine the role of BARD1 phosphorylation in this checkpoint, we first needed to establish if BARD1 itself functions in this checkpoint. Hela cells were treated with two rounds of BARD1 (A or B) siRNA nearly 24 hrs apart, resulting in efficient knockdown of the target (Figure 11). BRCA1 siRNA was utilized as a positive control [11], while control siRNA treatment served as a negative control. siRNA-treated samples were then treated with 65 ng/mL nocodazole to arrest cells in prometaphase of the cell cycle. Approximately 18 hrs later, cells were either mock treated or exposed to 10 Gy of IR (protocol adapted from Huang et al., 2005 [11]). Cells were collected at 0, 2, and 4 hrs following IR/mock treatment and the G_2/mitotic population of each culture was measured by flow cytometric analysis. Our results indicate that, as expected, mock-treated cells show a high percentage of cells in G_2/M initially (at 0 hrs) (~90%) and this population decreases over time for all the samples, independent of siRNA treatment (Figure 12). Cells treated with IR following nocodazole exposure all resulted in an intact IR-induced mitotic exit checkpoint, showing > 90% cells in G_2/M at all samples at each measured time point (Figure 12). From our findings, it appears that neither BRCA1 nor BARD1 appear to have a role in the IR-induced mitotic exit checkpoint. Since BARD1 itself could not be implicated in the IR-induced mitotic exit checkpoint, we did not examine the role of BARD1 mitotic phosphorylations in this checkpoint.

DNA Damage Sensitivity

Clonogenic survival assays were employed to assess the role of BARD1 phosphorylation in cell sensitivity to DNA damaging agents, such as IR, MMC, and camptothecin. Following two rounds of control or BARD1 siRNA treatments, cells were replated at low density (~2000 cells/plate) followed by 0-4 Gy IR doses. After 10 days of culture, the cells were stained with Giemsa and drug-resistant colonies were counted. We observed that cells depleted of BARD1 show increased sensitivity to IR treatment (Figure 13, 14A), implying that BARD1 is required for cell survival following DNA double-strand breaks. BARD1-depleted cells were then reconstituted with either wild-type or mutant siRNA-resistant forms of BARD1. As seen in Figure 14A, reconstitution with wildtype siRNA-resistant BARD1 provides a complete rescue of cell survival after IR (Figure 14A). Reconstituting BARD1-depleted cells with siRNA-resistant BARD1 polypeptides bearing specific mitotic phosphorylation mutations (individual or in tandem) also resulted in a complete rescue of cell survival post-IR checkpoint, suggesting that mitotic phosphorylation of BARD1 is not required for this function (Figure 14A).

A similar experiment was performed with mitomycin (0-200 ng/mL), which introduces inter- and intra-strand crosslinks. Thus, BARD1-depeleted cells were replated at low density followed by 0-200 ng/mL mitomycin C treatment for 4 hr. After 10 days in culture, the cells were stained with Giemsa and drug-resistant colonies were counted. Our findings show that following BARD1 knockdown (Figure 13), there is a ~2-3 fold decrease in cell survival after MMC treatment compared to control treated samples (Figure 14B). Reconstitution with wild-type siRNA-resistant form of BARD1 yielded a partial rescue in survival (~50%) (Figure 14B). The level of rescue was reduced in cells reconstituted with BARD1 polypeptides bearing the S148A+T299A mutations, while BARD1 polypeptides with mutations of all seven mitotic sites mutated (S148, S184, S186, S251, T299, S391, T394) (pX7A) failed to rescue survival, yielding a viability curve similar to that of unreconstituted BARD1-depleted cells (Figure 14B). Thus, cells that express mutant forms of BARD1 that lack mitotic phosphorylation sites are hypersensitive to particular forms of genotoxic stress such as MMC, suggesting a specific role for BARD1 phosphorylation in the cellular response to this form of DNA damage.

Lastly, BARD1-deplet ed cells were subjec ted to camptothecin (CPT), a drug which inhibits DNA topo I, using a protocol as described by Huertas et la., 2008 [12]. We observed a steady dec rease in su rvival of cells subjected to increasing levels of camptothecin treatment (0-10000 nM) following BARD1 knockdown (Figure 13, 14C), s uggesting a role f or BARD1 itself in survival after camptothecin-specific damage. While the le vels of cell surviv al at low dos es of the drug (10-1000 nM) were rescued almost completely upon reconstitution wit h wild-type BARD1 (Figure 14C), reconstituti on with the various mutant forms of BARD1 led to reduc ed lev els of cell s urvival (F igure 14C). Therefore, we conclude that mitotic phosphor ylation of BARD1 is important for the cellular response to camptothecin-mediated DNA damage.

Mitotic Spindle Assembly

Recently, Joukov et al. [2] descri bed a novel role for the BRCA1/BARD1 heterodimer in mitotic spindle assemb ly during normal (i.e., undamaged) cell cycle progression. Utilizing both *Xenopus* egg extracts and siRNA-depleted He la cells, they attributed chromosome segr egation defects and micronuclei formation to BRCA1/BARD1 depletion [2]. Therefore, I used a similar knockdown approach to assess t he role of BARD1 mit otic phosphorylation in mitotic s pindle assembly. Thus, Hela cells were cotransfected with siRNA and cDNA, and mitotic spin dles were visualized by st aining with α-tubulin-specific antibodi es, followed by DAPI counterstaining. By microscopy, sample s were evaluated for abnormalities in metaphase, such as misaligned chromo somes, dis organized spindles, and multipolar spindles, and in anaphase and t elophase for lagging chromosomes and micronuclei (Figure 15). Cell s depleted of both BRCA1 and BARD1

exhibited the greatest incidence of mitotic defects during both metaphase (66%) and anaphase/telophase (54%)(data not shown), as compared to control siRNA-treated cells (29% abnormal metaphases and 22% abnormal anaphases/telophases) (Figure 16). Cells depleted of BARD1 only also displayed significant mitotic defects (45% abnormal metaphases and 46% abnormal anaphases/telophases) (Figure 16). Reconstitution with wild-type BARD1 rescued the defective mitotic spindle phenotype successfully, resulting in 29% abnormal metaphases and 25% abnormal anaphases/telophases (Figure 16), comparable to that of control siRNA-treated cells. Reconstitution experiments with phospho-mutant forms of BARD1 are currently underway to determine the role of BARD1 mitotic phosphorylation in mitotic spindle assembly.

Homology-directed Repair

Previous studies have established that both BRCA1 and BARD1 are required for homology-directed repair (HDR) of double-strand DNA breaks (DSB) [13,14,3]. To evaluate the role of BARD1 phosphorylation in this process, Bard1-null mouse mammary tumor cells (cell line #2-218) bearing an HDR reporter construct (DR-GFP) were transfected with expression vectors encoding either wildtype or mutant forms of human BARD1 [3]. We previously showed that transfection of these cells with wild-type human BARD1 induces an approximately 5-fold increase in HDR function [3]. To examine the role of BARD1 phosphorylation in HDR, we generated expression plasmids encoding BARD1 in which 5 of the 7 mitotic phosphorylation sites (S184, S186, S251, S391, T394) were replaced with alanines (pX5A) (Figure 17). We also generated a phospho-mimicking mutant in which the same five residues were replaced with aspartic acid (pX5D). In addition, BARD1 polypeptides in which all seven mitotic sites (S148, S184, S186, S251, T299, S391, T394) were mutated to either alanine (pX7A) or aspartic acid (pX7D) were also evaluated (Figure 17). A plasmid bearing the deletion of the BRCT domain in BARD1 (dBRCT) served as a control as this construct is known to be defective in rescuing the HDR defect of these cells [3]. As shown in Figure 18, each of the phospho-mutant forms of BARD1 rescued the HDR defect of Bard1-null cells to a comparable extent as wild-type. Therefore, although BARD1 itself is required for HDR of DSBs, mitotic phosphorylation of BARD1 appears to be dispensible for this function.

KEY RESEARCH ACCOMPLISHMENTS

- Two different siRNAs were developed for specific depletion of BARD1 expression in human cell lines (Figures 1 and 5).
- BARD1 depletion in 293 cells impaired the IR-induced G_2 accumulation checkpoint defect (Figure 2), indicating that BARD1, like BRCA1, is required for this checkpoint.
- Reconstitution of BARD1-depleted cells with exogenous BARD1 polypeptides bearing mutations of specific mitotic phosphorylation sites fully restored the IR-induced G_2 accumulation checkpoint defect (Figures 3

and 4), indicating that mitotic phosphorylation of BARD1 is dispensable for this checkpoint.

- BARD1 depletion in Hela cells impaired the IR-induced transient G_2/M checkpoint (Figures 5 and 6), indicating that BARD1, like BRCA1, is required for this checkpoint.
- BARD1 (or BRCA1) depletion did not impair either the mitotic spindle checkpoint or the IR-induced mitotic exit checkpoint (Figures 8-12).
- Hela cells subjected to IR stress following BARD1 knockdown show a ~2-3 fold decrease in survival compared to control cells (Figure 13). Reconstitution of BARD1-depleted cells with BARD1 polypeptides bearing mutations of specific mitotic phosphorylation sites does not impair cell survival in response to IR damage (Figure 14A), indicating that BARD1 mitotic phosphorylation does not influence cell viability in response to IR.
- Cells depleted of BARD1 are sensitive to MMC treatment, exhibiting a ~2-3 fold decrease in cell survival (Figures 13 and14B). BARD1-depleted cells that were reconstitution with BARD1 polypeptides bearing phospho-mutations show a defect in survival after MMC treatment (Figure 14B), indicating that BARD1 mitotic phosphorylation is required for resistance to MMC, and as such may influence the cellular response to DNA cross-linking agents.
- BARD1-depleted cells reconstituted with BARD1 polypeptides bearing all seven phospho-mutations (pX7A) show an impairment in survival after camptothecin treatment (Figure 14C).
- BARD1 depletion led to a mitotic defects, particularly during metaphase and anaphase (Figures 15 and 16).
- Phospho-mutant and phospho-mimicking forms of BARD1 both rescue the HDR defect comparably to the wild-type form of BARD1 in BARD1-null cells (Figures 17 and 18), indicating that mitotic phosphorylation of BARD1 does not function in this form of DNA repair.

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(* Equal contribution and authorship)

Laufer, M., S.V. Nandula, **A.P. Modi**, S.Wang, M.Jasin, V. V. V. S. Murty, T.Ludwig, and R. Baer (2007). *Structural Requirements for the BARD1 Tumor Suppressor in Chromosomal Stability and Homology-directed DNA Repair.* J. Biol. Chem. 282: 34325-34333.

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Modi, A.P., A.D. Choudhury, and R. Baer (2007). *Functional Analysis of Mitotic Phosphorylations in BARD1.* American Association for Cancer Research (AACR) Annual Meeting (April 2007; Los Angeles, CA).

CONCLUSION

Using siRNA-mediated knockdown, we conclude that BARD1 is required for both the IR-induced G_2 accumulation and transient G_2/M checkpoints. However, phosphorylation of BARD1 does not impact the G_2 accumulation check point. Additionally, we have found that BARD1 knockdown does not impair the spindle assembly or the IR-induced mitotic exit checkpoints. Cells void of BARD1 are to a variety of DNA stressors, including IR, mitomycin C (MMC), and camptothecin. Although mitotic phosphorylation of BARD1 was dispensable for survival following IR damage, it appears to be required for effective cellular resistance to both MMC and camptothecin. Cells depleted of BARD1 show abnormalities in mitotic spindle assembly, with an increase in disorganized and multipolar spindles in metaphase as well as lagging chromosomes and micronuclei in anaphase and telophase; Reconstitution analyses currently underway should elucidate the role of mitotic phosphorylation in mitotic spindle assembly. Finally, although BRCA1 and BARD1 are both required for homology-directed repair of double-strand DNA breaks, phosphorylation of BARD1 appears to be dispensable for this process.

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APPENDICES

Abstracts:

Modi, A.P. and R. Baer (2008). *Functional Analysis of Mitotic Phosphorylations in BARD1.* Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope 2008 Meeting (June 2008; Baltimore, MD).

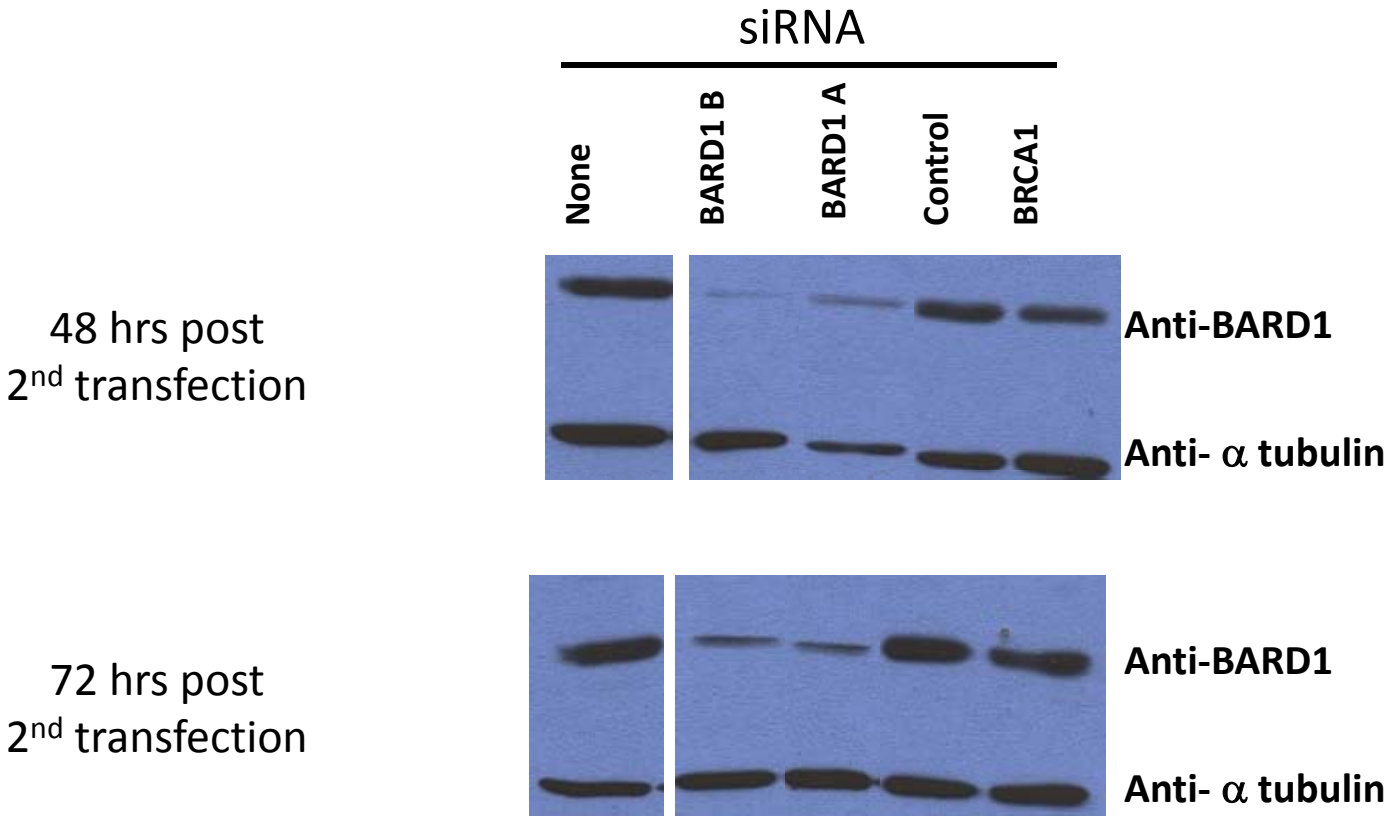
The BRCA1 tumor suppressor has been implicated in numerous cellular processes, including DNA repair, cell cycle checkpoint control, and mitotic spindle assembly. *In vivo*, BRCA1 exists in association with BARD1 and the BRCA1/BARD1 heterodimer is thought to mediate many BRCA1 functions, including its tumor suppression activity. These functions may be regulated in part by post-translational modifications of the heterodimer. We previously showed that the phosphorylation state of BARD1 is cell cycle regulated and that BARD1 is hyperphosphorylated at seven distinct sites during mitosis. The goals of this study are to evaluate the role of BARD1 phosphorylation in cell cycle checkpoints and DNA repair pathways that are dependent on BRCA1, such as the ionizing radiation (IR)-induced G_2 accumulation checkpoint and homology-directed repair (HDR) of double-strand DNA breaks (DSB).
To study the function of BARD1 phosphorylation in the IR-induced G_2 accumulation checkpoint, we used BARD1-specific siRNAs to reduce the expression of endogenous BARD1 in 293 cells and then restored expression with siRNA-resistant wildtype or mutant forms of exogenous BARD1. Western blot analysis indicates that two sequential siRNA transfections with either of two distinct siRNAs resulted in >90% knockdown of endogenous BARD1 and a substantial defect in the IR-induced G_2 accumulation checkpoint. This result confirms that BARD1, like BRCA1, is required for activation of this cell cycle checkpoint. Moreover, partial rescue of the checkpoint was achieved upon co-transfection of the siRNA-treated cells with expression vectors encoding siRNA-resistant forms of exogenous wild-type BARD1 mRNA. In addition, checkpoint function was also rescued to a comparable degree with expression vectors encoding siRNA-resistant BARD1 mRNA bearing mutations of the seven specific phosphorylation sites. These results indicate that mitotic phosphorylation of BARD1 is not required for its role in activation of the IR-induced G_2 accumulation checkpoint defect. We are currently

applying this same approach to determine the function of BARD1 phosphorylation in other BRCA1-dependent IR-induced checkpoints, such as the transient G/M checkpoint, the decatenation checkpoint, and the mitotic exit checkpoint.
To evaluate the function of BARD1 phosphorylation in homology-directed repair (HDR) of double-strand DNA breaks (DSB), Bard1-null mouse mammary tumor cells bearing an HDR reporter construct (DR-GFP) were transfected with expression vectors encoding either wildtype or mutant forms of human BARD1. Briefly, this reporter contains two distinct nonfunctional copies of the GFP gene: one copy (*Sce*GFP) is disrupted by the recognition site for the rare-cutting endonuclease I-*Sce*I, while the other copy (*i*GFP) encodes only an internal region of GFP. However, a functional GFP gene can be regenerated when a DSB break triggered by I-*Sce*I cleavage of the SceGFP is repaired by HDR utilizing *i*GFP as a template, and such events can be quantified by flow cytometry. Using this assay, we previously showed that transfection of these Bard1-null cells with an expression vector encoding human BARD1 induces an approximately 5-fold increase in HDR function. However, a similar increase in HDR function was readily achieved upon transfection with expression vectors encoding phospho-mimicking and phospho-deficient forms of BARD1, indicating that mitotic phosphorylation of BARD1 is not required for HDR of DSBs.

Modi, A.P., A.D. Choudhury, and R. Baer (2007). *Functional Analysis of Mitotic Phosphorylations in BARD1.* American Association for Cancer Research (AACR) Annual Meeting (April 2007; Los Angeles, CA).

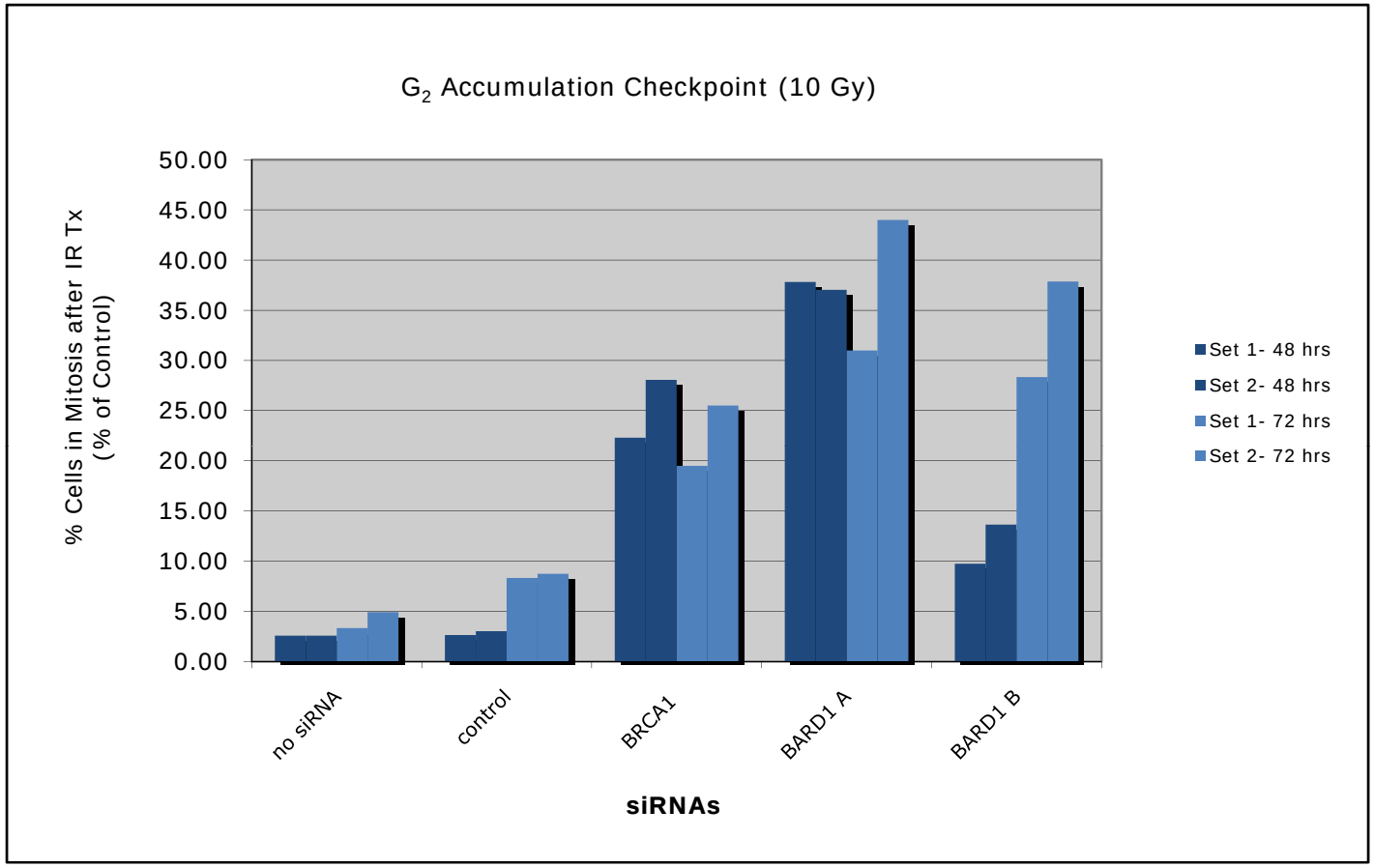
The breast and ovarian-specific tumor suppressor BRCA1 has been implicated in numerous cellular processes, including DNA repair, cell cycle checkpoint control, and mitotic spindle assembly. *In vivo*, BRCA1 primarily exists in association with BARD1 and the BRCA1/BARD heterodimer is thought to mediate the tumor suppression activity of BRCA1. It has been previously shown that the phosphorylation state of the BARD1 polypeptide is cell cycle regulated and that BARD1 is hyperphosphorylated in mitosis. Seven mitotic phosphorylation sites have been identified within BARD1, two of which, S148 and T299, occur within cdk consensus motifs. To study the functional consequences of mitotic BARD1 phosphorylation, we utilized an siRNA-mediated approach to knockdown endogenous BARD1 expression and then restored expression with siRNA-resistant exogenous wild-type or mutant forms of BARD1. In this manner, we are evaluating the role of BARD1 mitotic phosphorylation in cell cycle checkpoint control and spindle assembly.

Figure 1: Knockdown of BARD1 in 293 Cells



Following siRNA treatment, BARD1 endogenous levels are efficiently reduced at both 48 hrs and 72 hrs post- 2nd siRNA transfection.

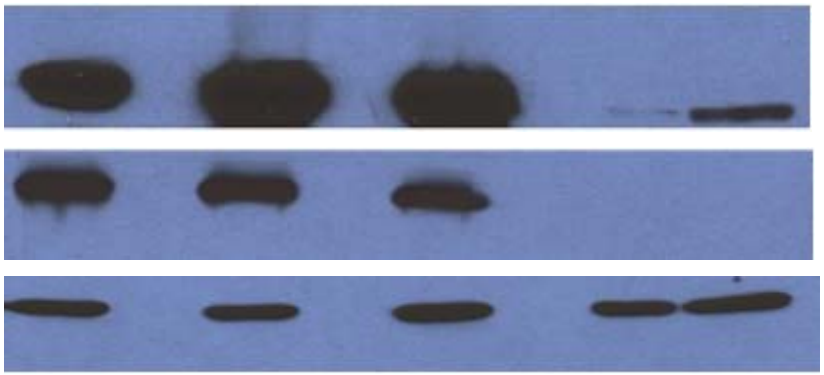
Figure 2: G_2 Accumulation Checkpoint in 293 Cells



After IR treatment, cells with a functional G_2 accumulation checkpoint will arrest in G_2 and not progress to mitosis. BARD1 knockdown with siRNAs also results in a $G2_2$ accumulation checkpoint defect.

Figure 3 : Rescue of siRNA Knockdown in 293 Cells

siRNA	B	B	B	B	Control
wt- BARD1 B SM cDNA			+		
*mut 1- BARD1 B SM cDNA		+			
*mut 2- BARD1 B SM cDNA	+				



Anti-BARD1

Anti-Flag

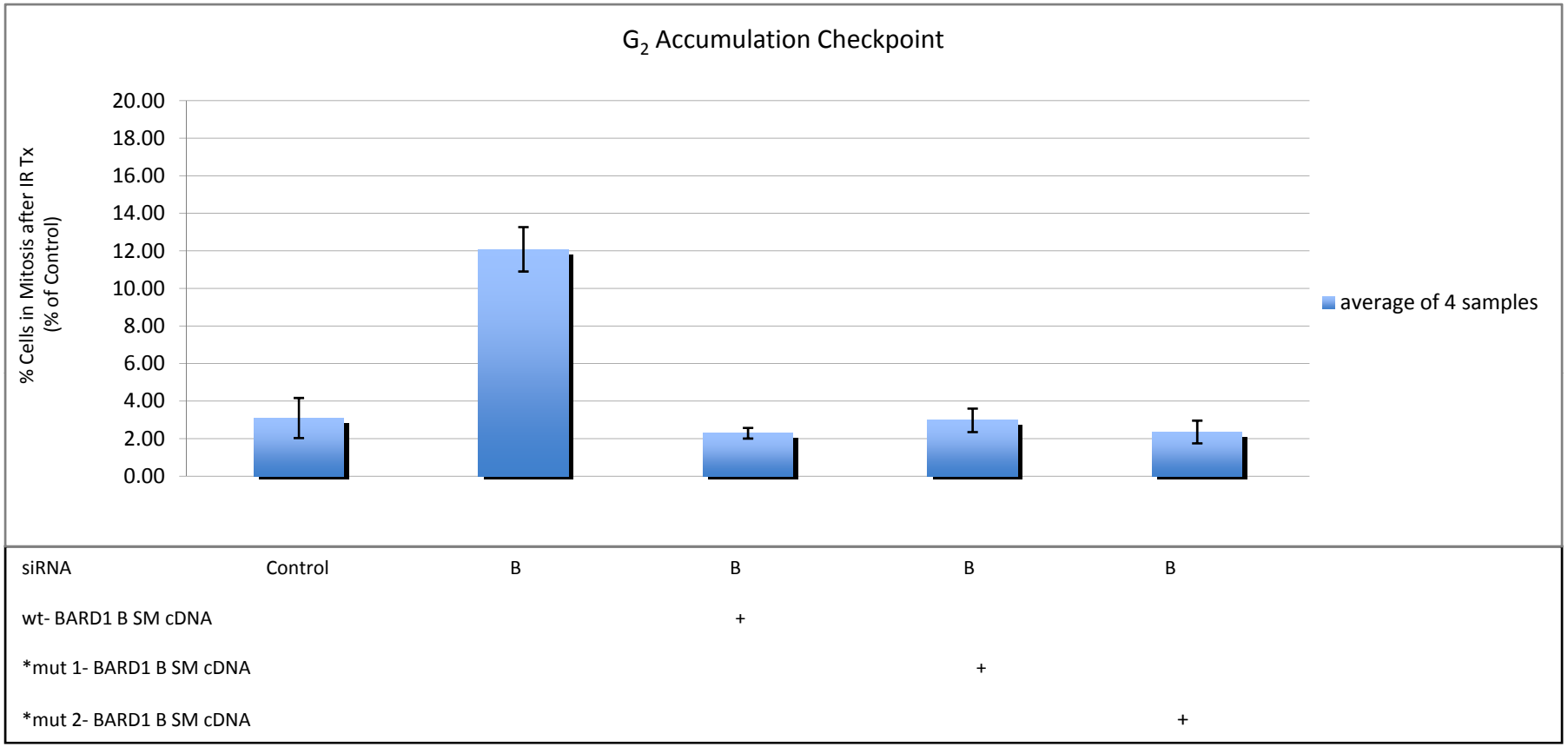
Anti- α tubulin

*mutant 1 = S148A +T299A

*mutant 2 = S184A, S186A, S251A, S391A, T394A

Reconstitution of cells with siRNA-resistant constructs results in overexpression of BARD1 compared to endogenous levels (control siRNA treated lane).

Figure 4 : G_2 Accumulation Checkpoint

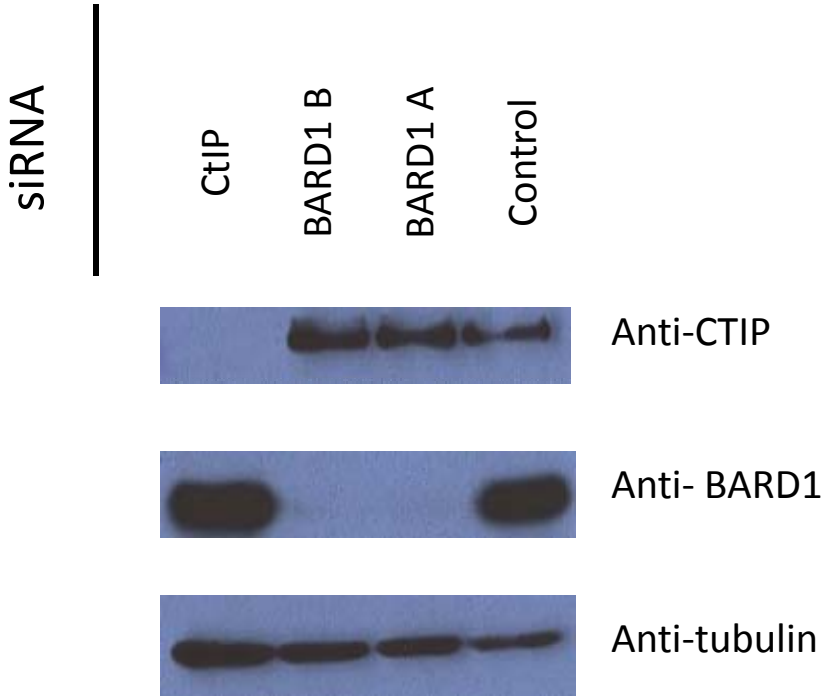


*mutant 1 = S148A +T299A

*mutant 2 = S184A, S186A, S251A, S391A, T394A

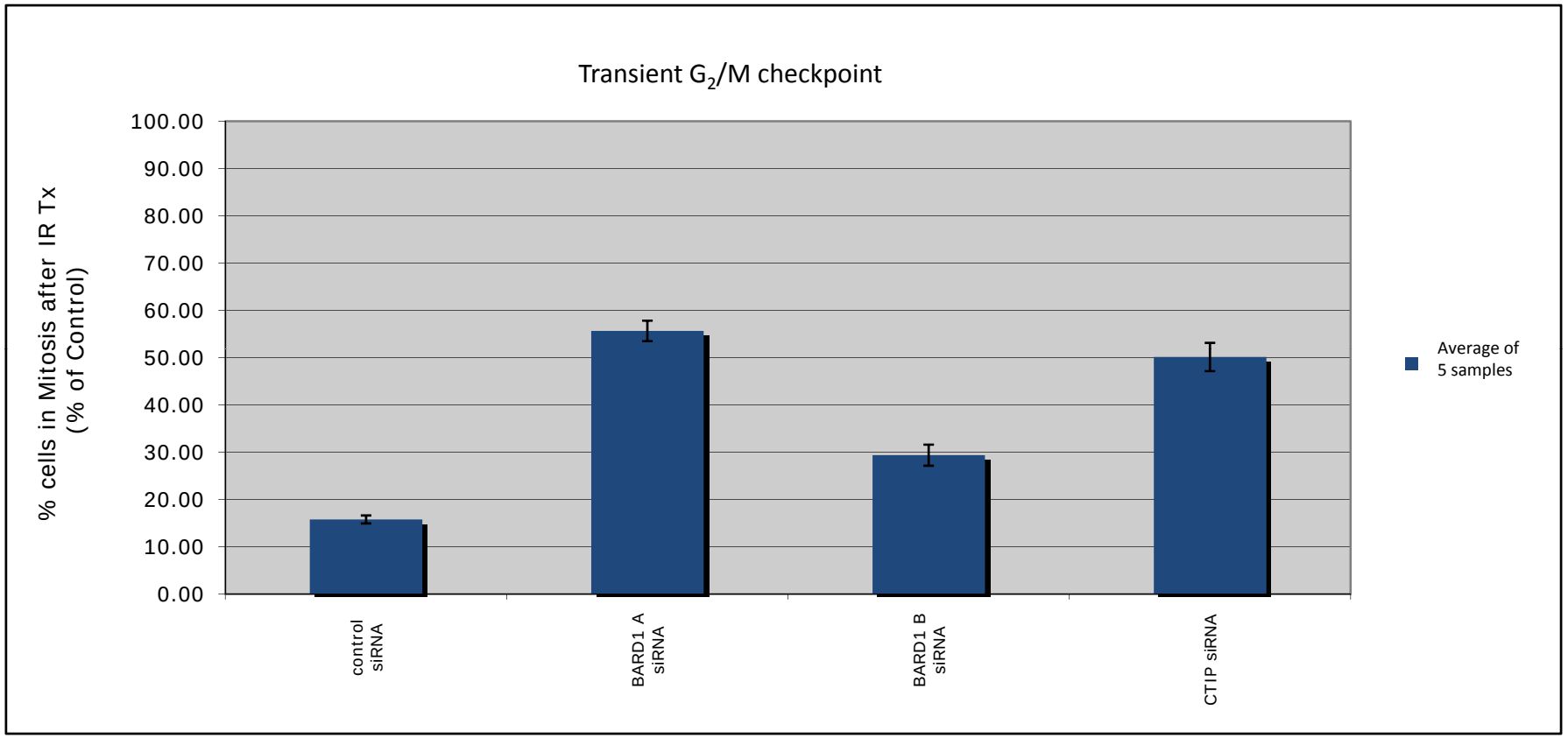
After IR treatment, cells treated with BARD1 B siRNA result in a defective G_2 accumulation checkpoint. Cells transfected with a wild-type siRNA-resistant form of BARD1 or phosphomutant forms all result in an intact checkpoint, suggested that mitotic phosphorylations of BARD1 do not function in this checkpoint.

Figure 5: Knockdown of BARD1 in Hela cells



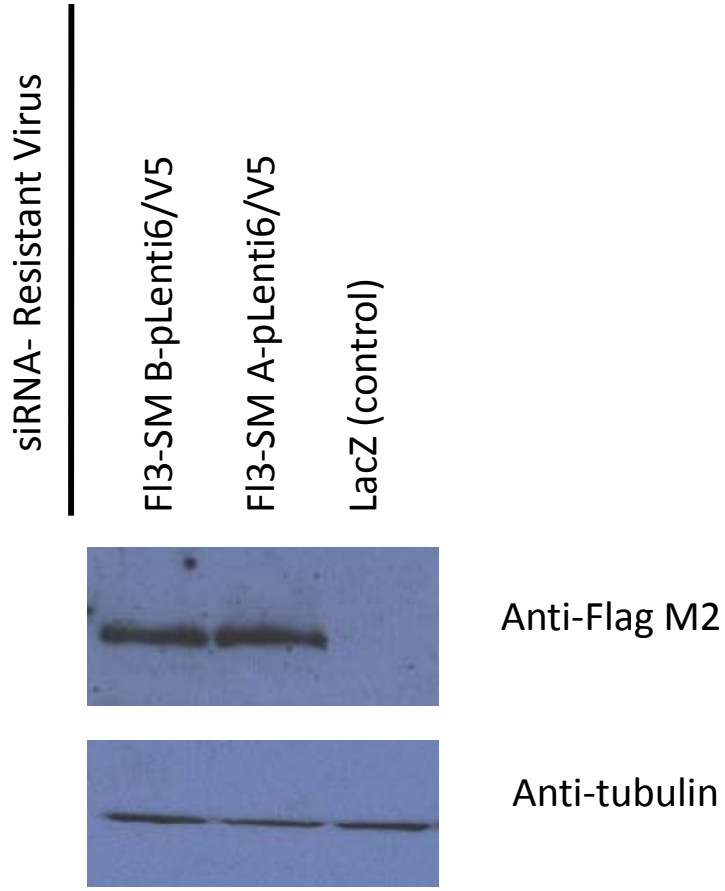
Efficient knockdown of BARD1 is observed with both BARD1 A and B siRNAs. CtIP siRNA also knockdowns its intended target.

Figure 6: Transient G_2/M Checkpoint in Hela Cells



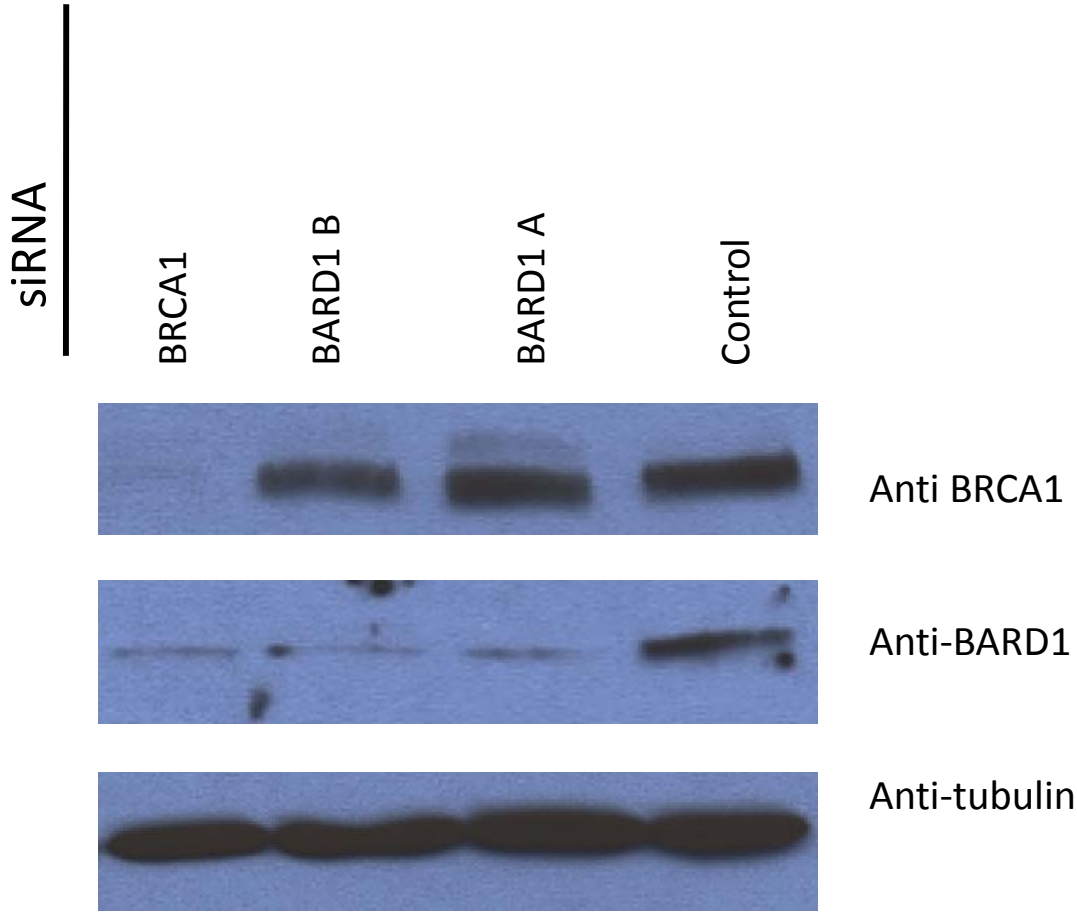
After IR treatment, cells with a functional transient G_2/M checkpoint will arrest in G_2 and not progress to mitosis. BARD1 knockdown with siRNAs also results in a transient G_2/M checkpoint defect. As a positive control CtIP siRNA was utilized, since it is known to function in this checkpoint.

Figure 7: Infection of BARD1 siRNA-Resistant Lentivirus in Hela cells



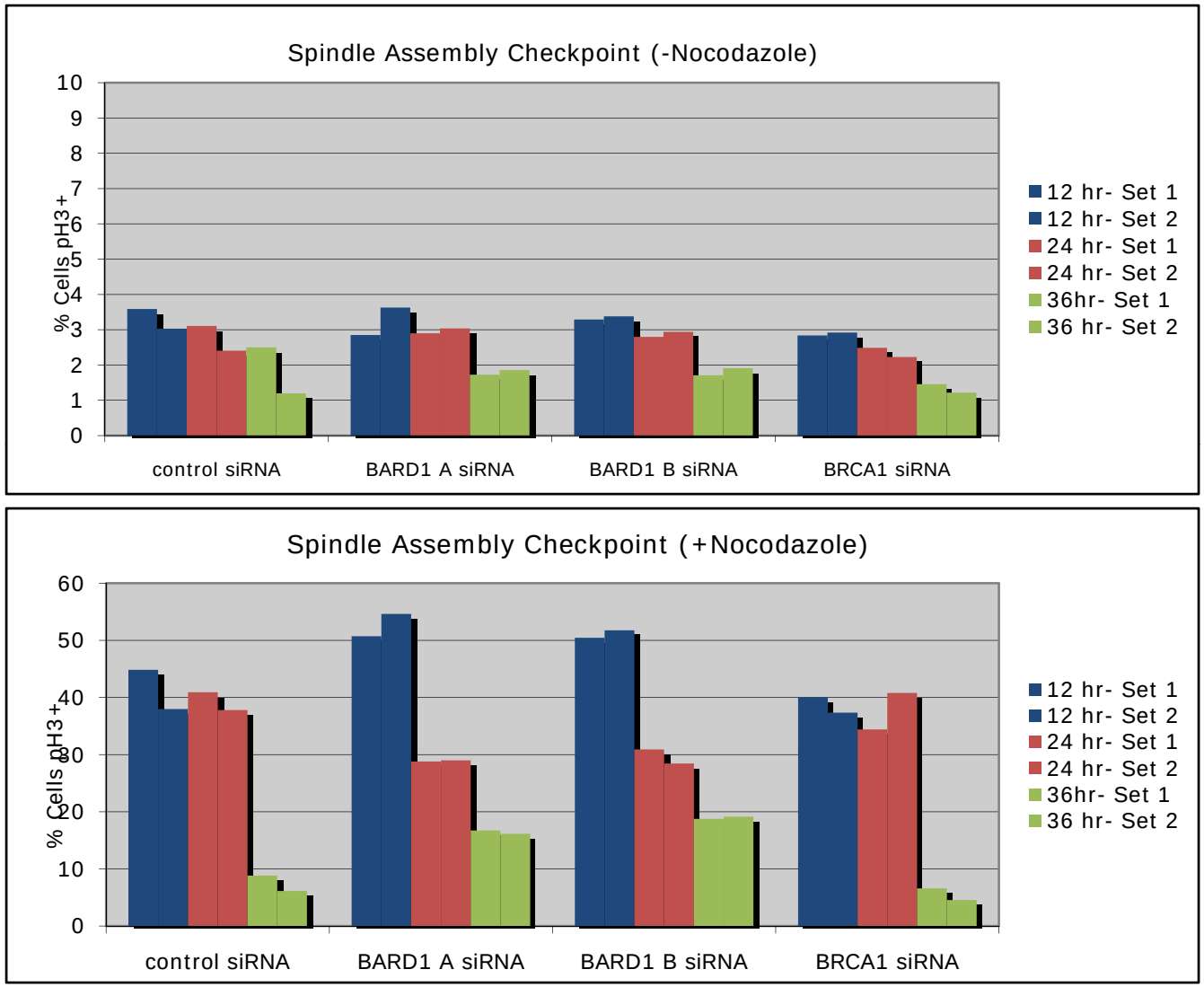
Flag-tagged (Fl3) lentiviruses created for resistant forms of BARD1 against both BARD1 A and B siRNAs express efficiently in Hela cell line.

Figure 8 : Knockdown of BARD1 in 293 cells (Spindle Assembly Checkpoint)



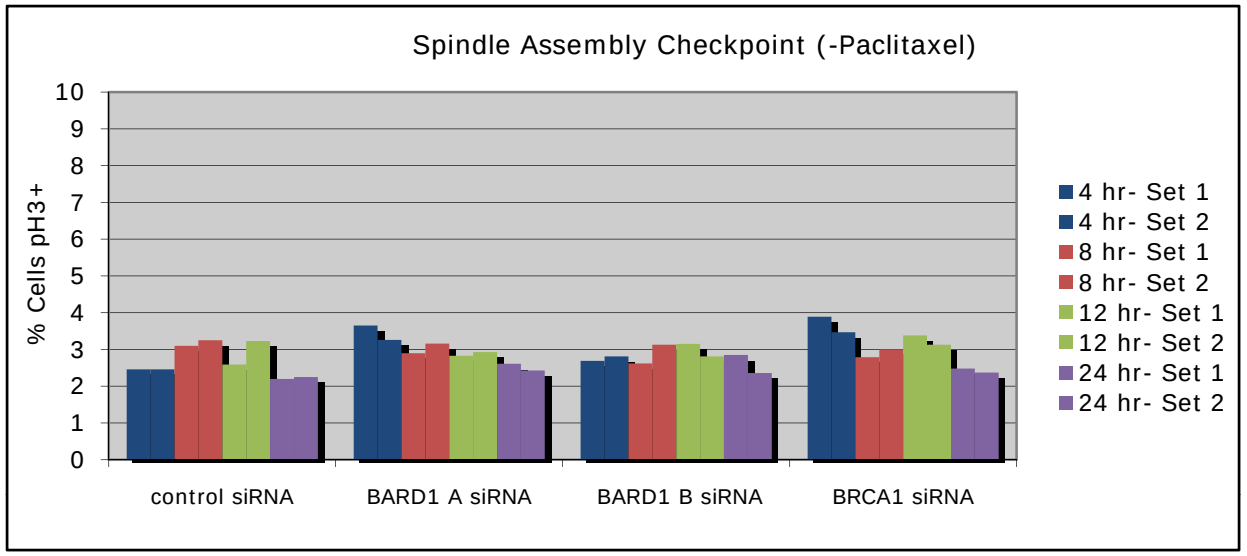
Knockdown of BARD1 and BRCA1 is efficient in 293 cell line (for purposes of analyzing the spindle assembly checkpoint).

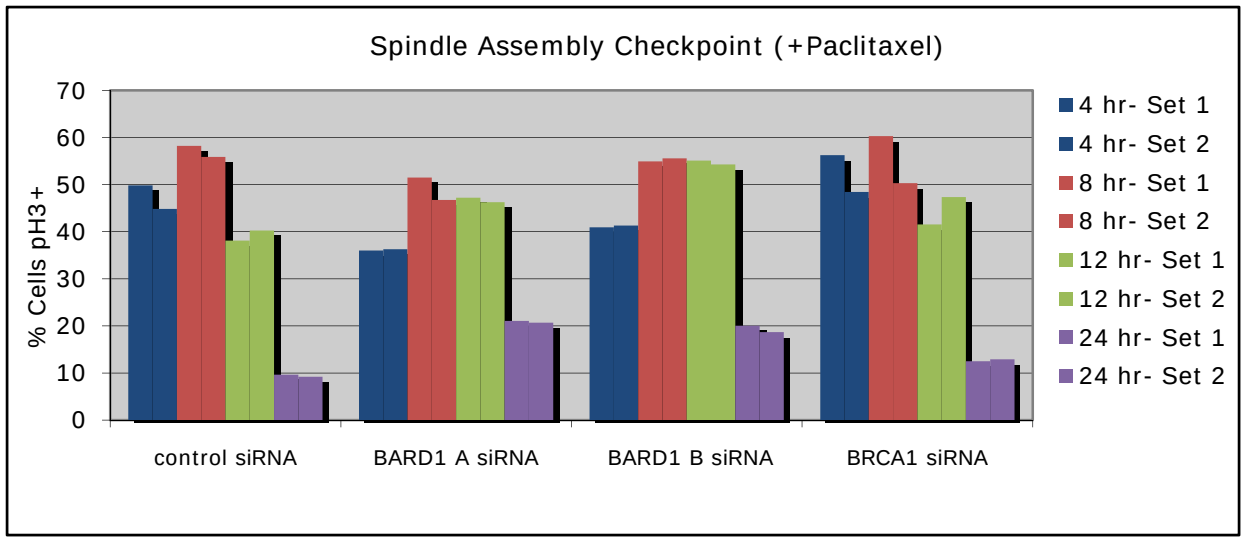
Figure 9 : Spindle Assembly Checkpoint I



Treatment of BARD1 or BRCA1 knockdown cells with nocodazole does not induce a defective spindle assembly checkpoint.

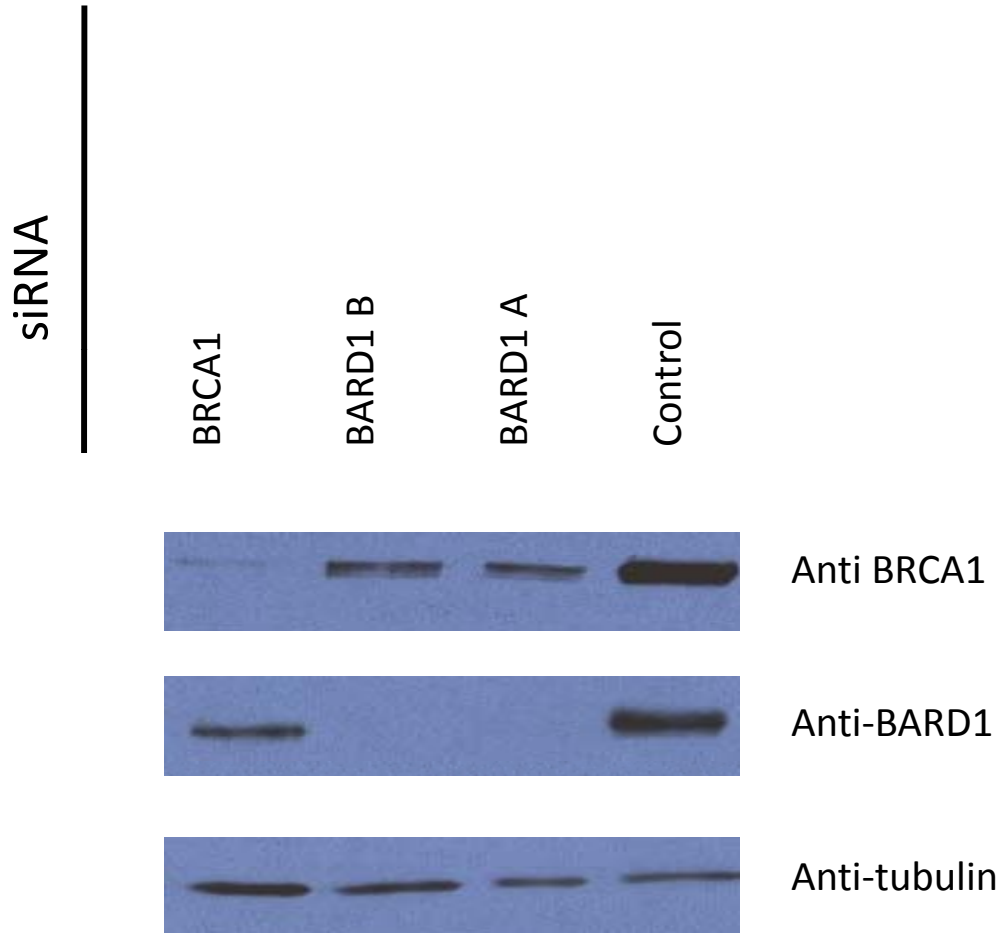
Figure 10 : Spindle Assembly Checkpoint II





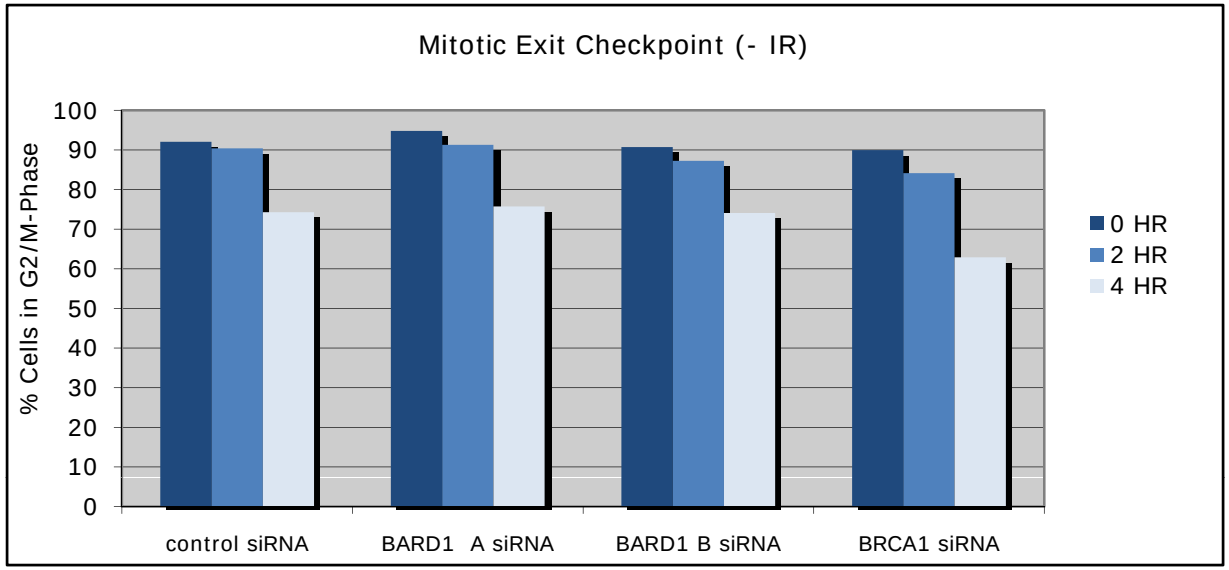
Cells treated with BARD1 siRNAs, followed by paclitaxel does not induce a defective spindle assembly checkpoint, indicating that BARD1 does not function in this checkpoint role.

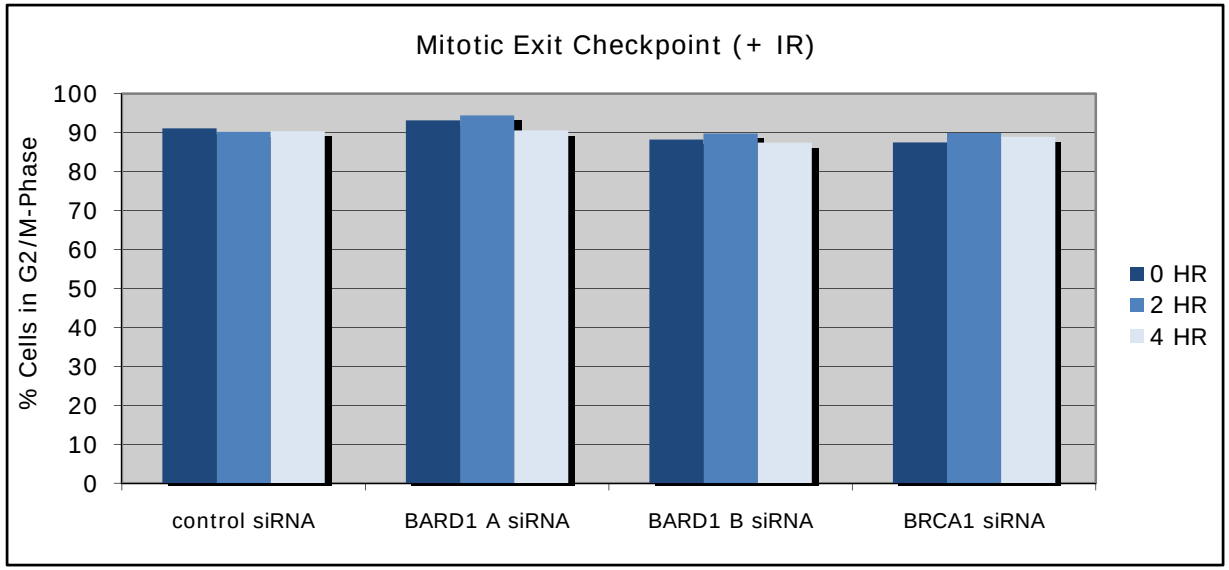
Figure 11 : Knockdown of BARD1 in Hela cells (Mitotic Exit Checkpoint)



Hela cells were targeted with BARD1 or BRCA1 siRNAs for analysis of the IR-mediated mitotic exit checkpoint.

Figure 12 : Mitotic Exit Checkpoint

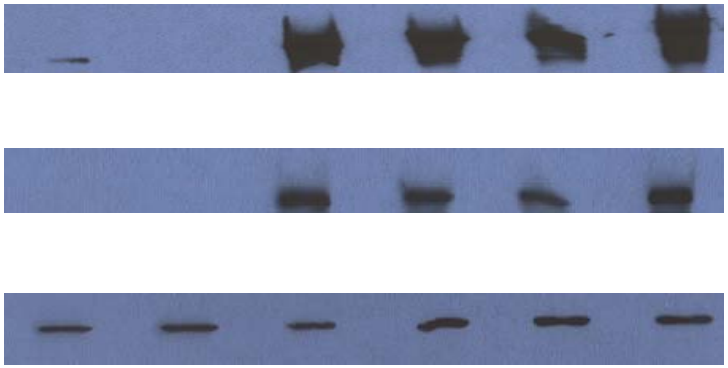




An intact IR-induced mitotic exit checkpoint occurs following BARD1 knockdown, suggesting BARD1 does not function in this checkpoint.

Figure 13 : Rescue of siRNA Knockdowns for DNA Damage Survival Assays

siRNA	Control	B	B	B	B	B
wt- BARD1 B SM cDNA			+			
*mut 1- BARD1 B SM cDNA				+		
*mut 2- BARD1 B SM cDNA					+	
*mut 3-BARD1 B SM cDNA						+



*mutant 1 = S148A +T299A

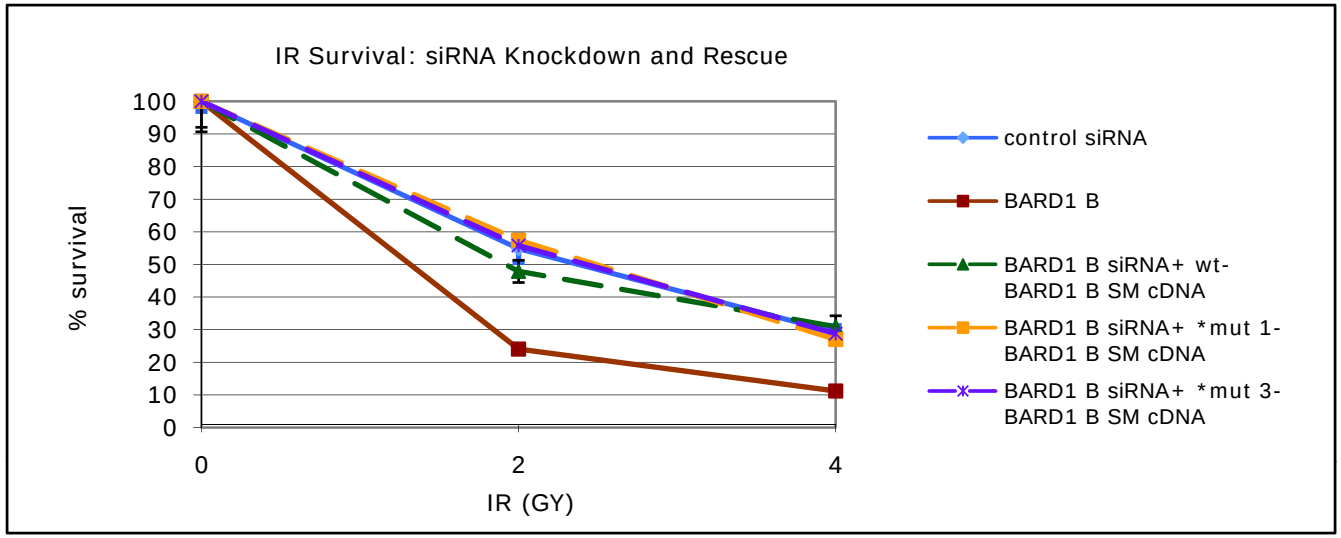
*mutant 2 = S184A, S186A, S251A, S391A, T394A

*mutant 3= S148A, S184A, S186A, S251A, T299A, S391A, T394A

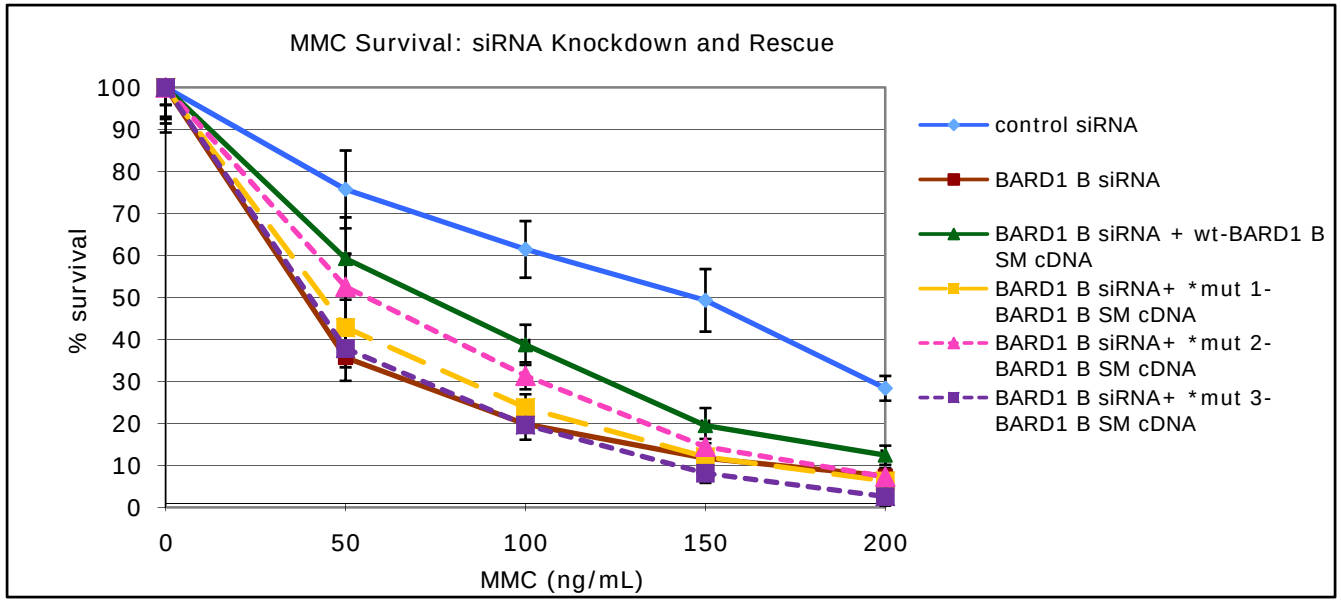
Hela cells were knocked down with BARD1 B siRNA and then reconstituted with Flag-tagged wild-type or phosphomutant forms of the resistant construct.

Figure 14 : Rescue of siRNA Knockdowns for DNA Damage Survival Assays

A.



B.



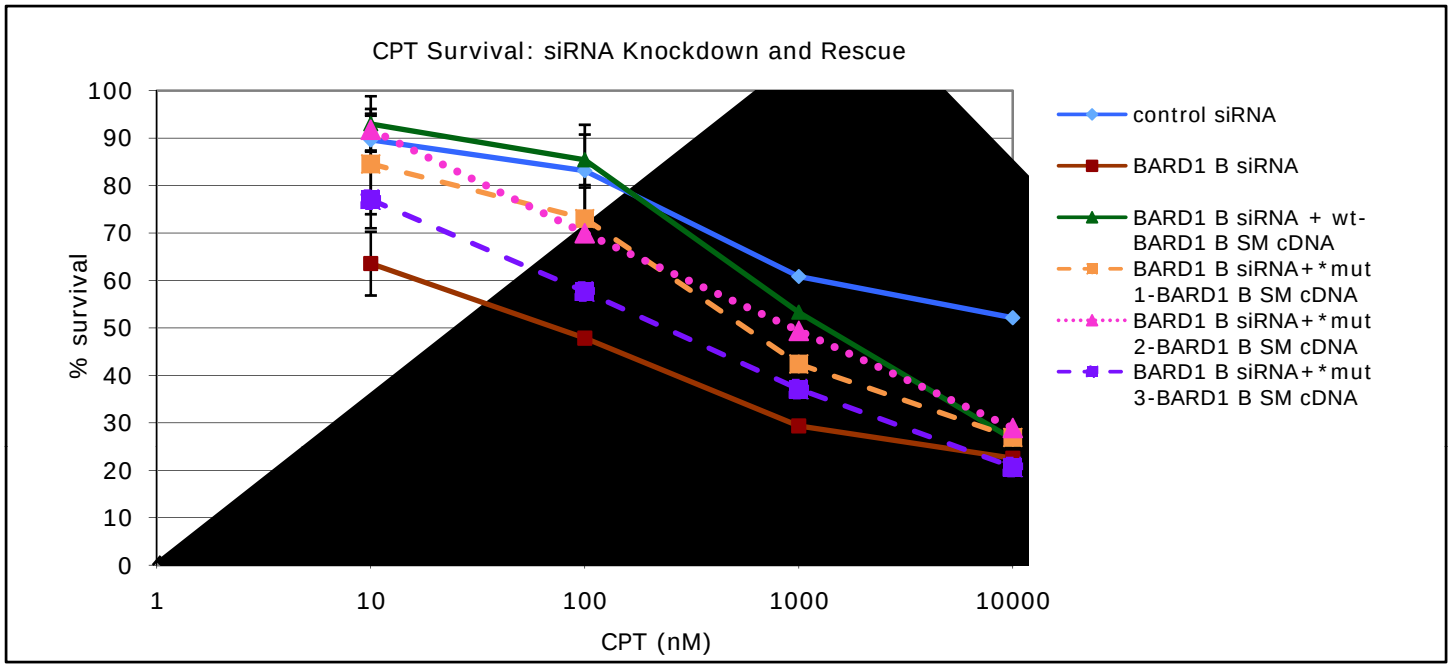
*mutant 1 = S148A +T299A

*mutant 2 = S184A, S186A, S251A, S391A, T394A

*mutant 3= S148A, S184A, S186A, S251A, T299A, S391A, T394A

Figure 14 : Rescue of siRNA Knockdowns for DNA Damage Survival Assays

C.



*mutant 1 = S148A +T299A

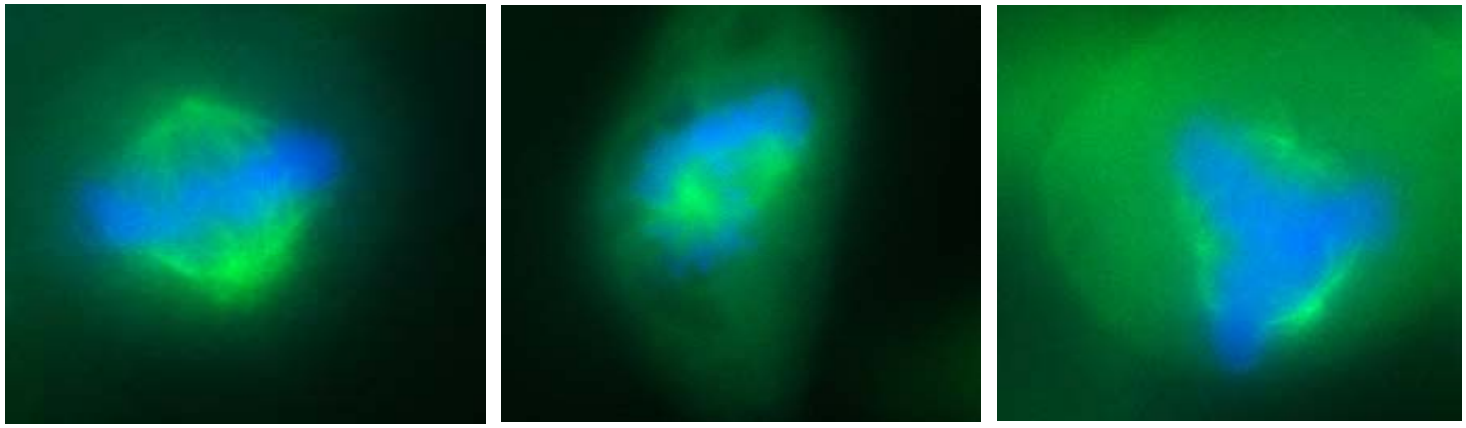
*mutant 2 = S184A, S186A, S251A, S391A, T394A

*mutant 3= S148A, S184A, S186A, S251A, T299A, S391A, T394A

BARD1 knockdown cells treated with IR, MMC, or CPT all exhibit an impairment in cell survival. Following MMC or CPT treatment, BARD1 siRNA treated samples reconstituted with phosphomutant siRNA-resistant forms of BARD1 show a decrease in cell survival compared to cells rescued with the wild-type resistant form, suggesting a role for BARD1 mitotic phosphorylations in cellular resistance towards particular forms of DNA damage.

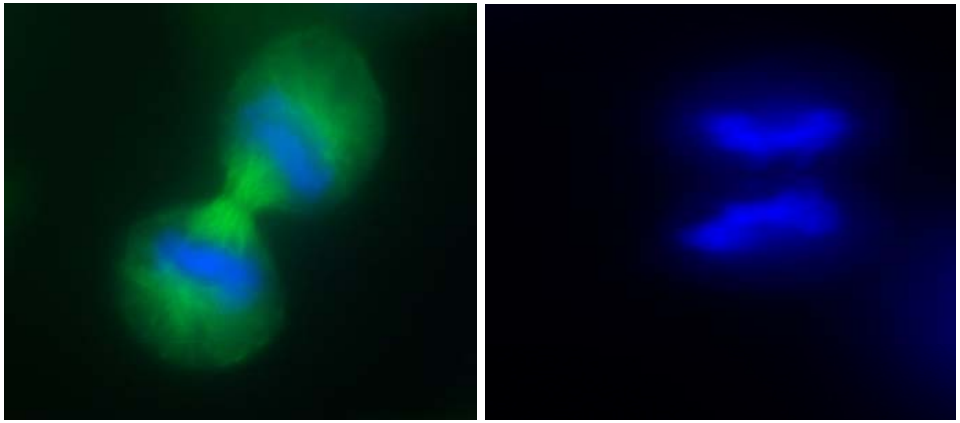
Figure 15 : Mitotic Images

A.



I II III

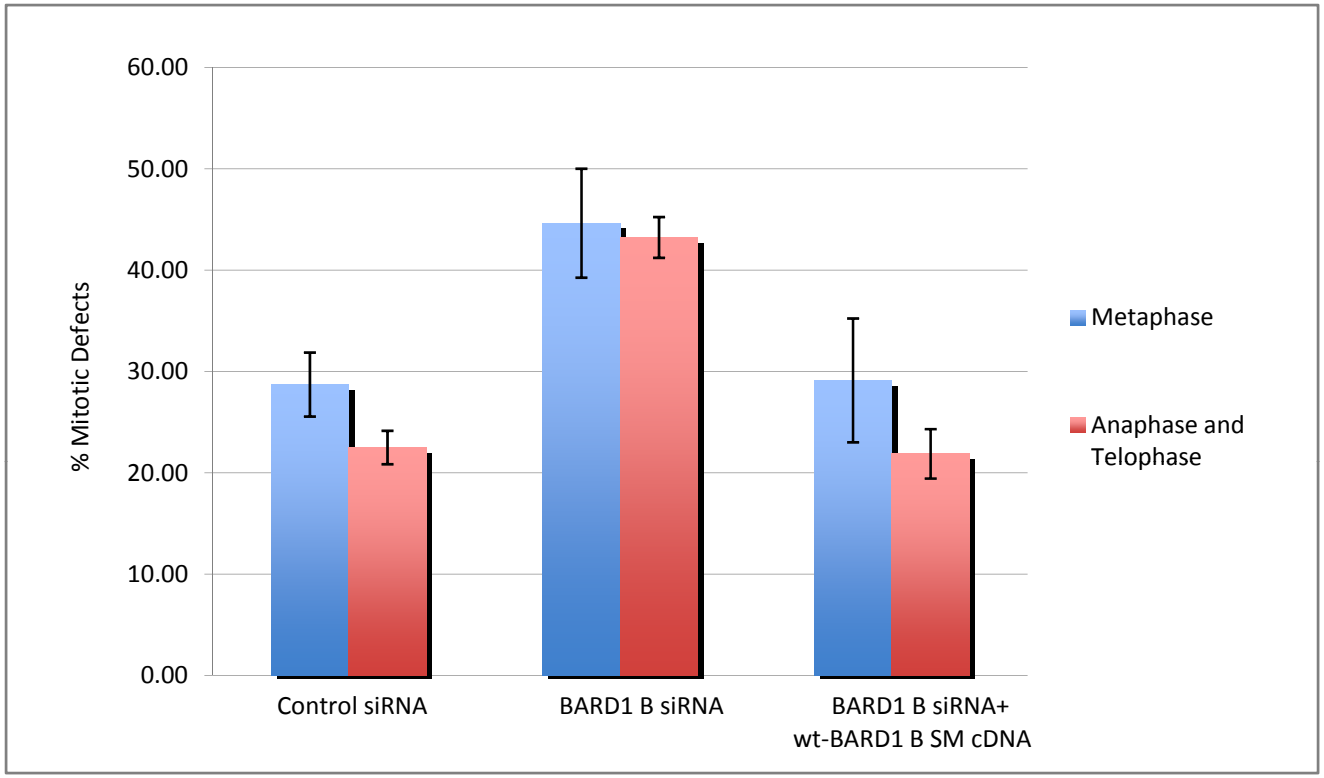
B.



IV V

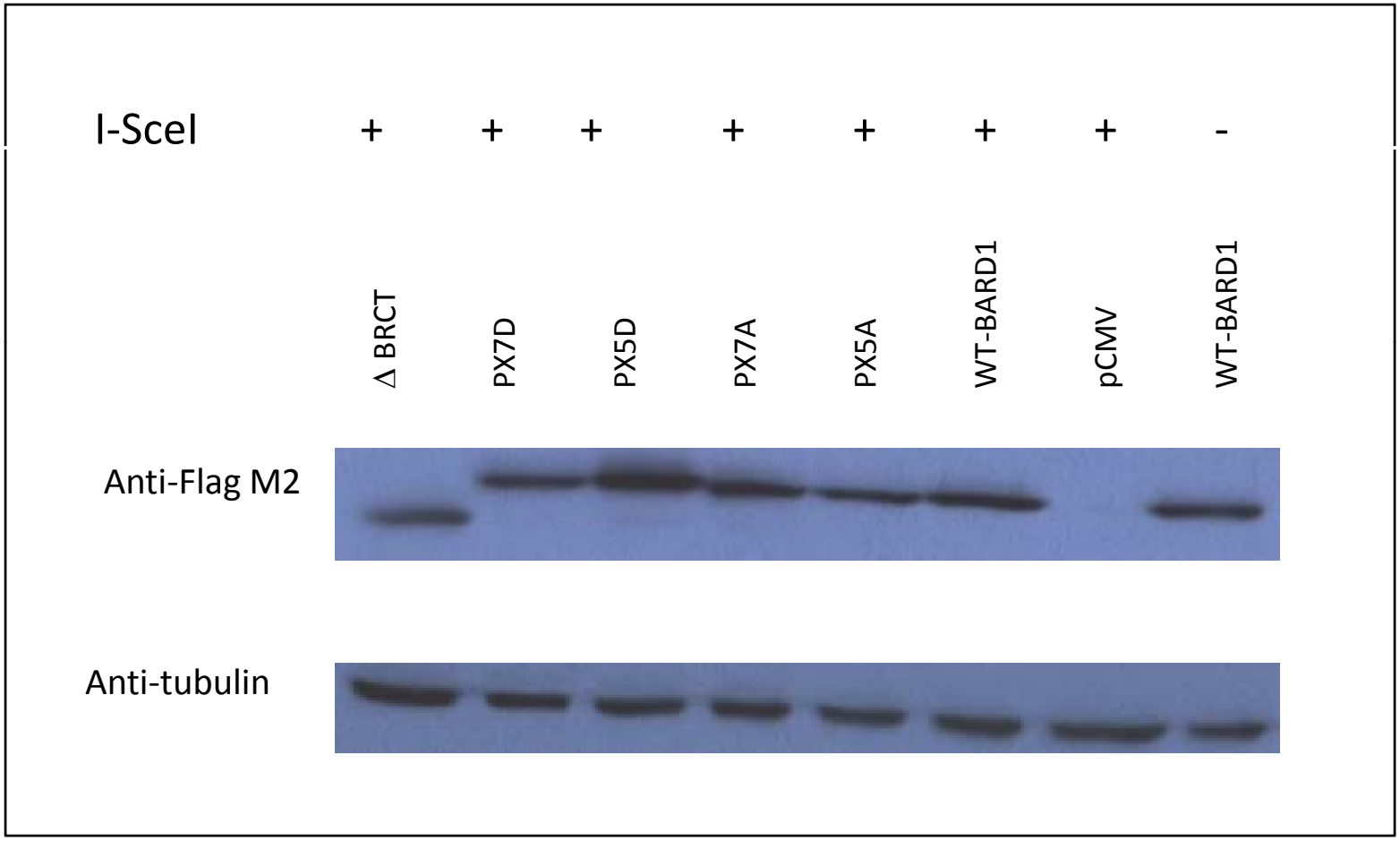
Image I depicts a normal metaphase, with chromosomes (blue) aligned in the center. Image II shows an abnormal spindle, with disorganized chromosomes. Image III displays a multipolar spindle, another abnormality observed in metaphase. Image IV depicts a normal anaphase-telophase, while Image V displays lagging chromosomes found in abnormal anaphases.

Figure 16 : Mitotic Spindle Assembly



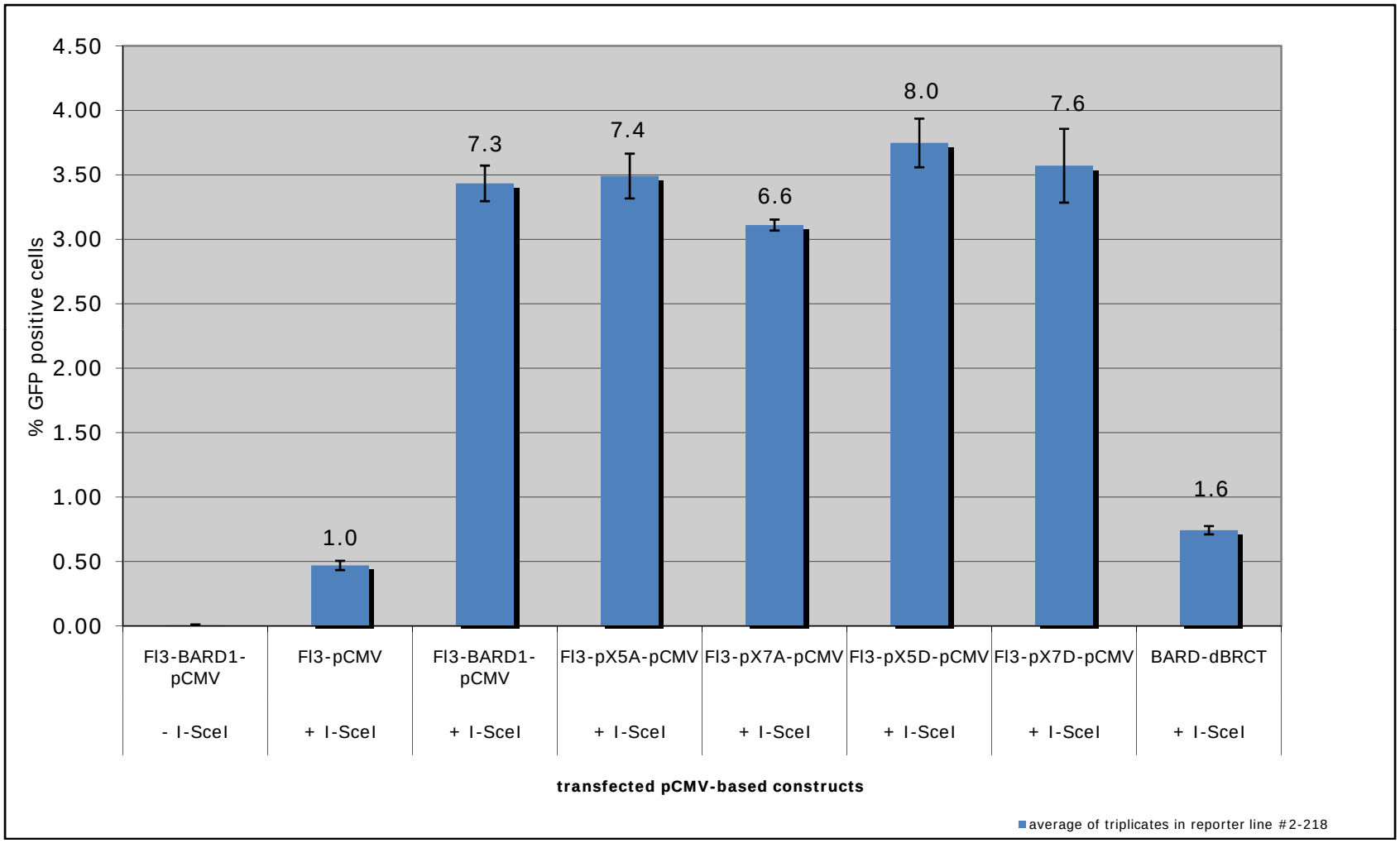
Following BARD1 siRNA treatment (western blot not shown), cells exhibit an increase in abnormal mitosis as measured by immunoflorescence. Rescue with the siRNA-resistant form of BARD1 decreases abnormalities to levels of control siRNA-treated samples.

Figure 17: Transfection of Constructs in Bard1-null cells



Bard1-null cells transfected with Flag-tagged constructs bearing wild-type, phosphomutant or phosphomimicking forms of BARD1 were analyzed for comparable transfection levels using the Flag-M2 antibody. ΔBRCT construct was used a positive control, since BARD1 BRCT repeats are required for efficient HDR.

Figure 18: Rescue of Homology-directed repair defect in BARD1-null cells



Bard1-null cells transfected with Flag-tagged constructs bearing wild-type, phosphomutant or phosphomimicking forms of BARD1 all comparably rescue the HDR defect observed in Bard1-null cells, suggesting that mitotic phosphorylation of BARD1 does not function in this form of repair. ΔBRCT construct was used a positive control, since BARD1 BRCT repeats are required for efficient HDR.